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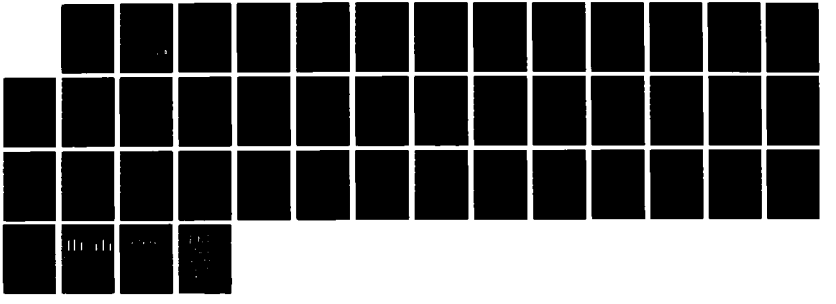

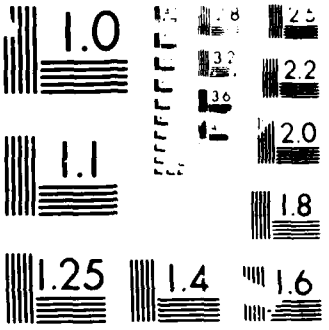
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OPERATION EVEREST II: ALTERATIONS IN THE IMMUNE SYSTEM AT HIGH ALTITUDE

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RUNNING HEAD: Hypoxia-Induced immune dysfunction

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Some of this study's results were presented as an abstract at the F.A.S.E.B. Meeting in St. Louis, Missouri, 1986.

ABSTRACT

We investigated the effects of progressive hypobaric hypoxia simulating an ascent to 25,000 ft (7,620m) over 4 weeks on immune function. Multiple simultaneous *in vitro* and *in vivo* immunologic parameters were obtained from 7 subjects at sea level, 7,500 ft (2,286m), and 25,000 ft during a decompression chamber exposure. PHA stimulated thymidine uptake and protein synthesis in mononuclear cells were reduced at extreme altitude. An increase in monocytes without changes in B cells or T cell subsets was also observed by flow cytometry analysis. Plasma IgM and IgA but not IgG levels were increased at altitude, whereas PWM stimulated *in vitro* IgG, IgA and IgM secretion was unchanged. *In vitro* PHA stimulated interferon production and NK cytotoxicity did not change statistically but large inter-subject differences were observed during exposure to 25,000 ft. Nasal wash IgA and lysozyme levels, and serum antibodies to nuclear antigens were not influenced by altitude exposure. These results suggest that T cell function is blunted during exposure to severe hypoxemia, whereas B cell function and mucosal immunity are not. While the mechanism of altered *in vitro* immune responsiveness following exposure to various environmental stressors has not been elucidated in humans, hypoxia may induce immune dysfunction as suggested by *in vitro* immune effector cell function assays.

INTRODUCTION

There are over 25 million inhabitants residing at altitudes above 10,000 feet. In addition, millions of lowland dwellers are afflicted with pulmonary or cardiac diseases resulting in chronic hypoxemia. To date, however, the only prospective studies investigating the effects of hypoxia on human immunobiology have been reported in the Soviet literature (1,2-7).

At higher terrestrial elevations, the reduced barometric pressure results in progressive arterial oxygen desaturation due to the lowered partial pressure of O_2. Hypoxemia invokes a variety of physiological responses which may facilitate O_2 delivery at the cellular level. In some individuals, however, these responses may prove maladaptive resulting in various forms of altitude illness (8).

Epidemiologic data provide indirect evidence that hypoxia may impair immune competence in humans. A higher incidence of pneumonia has been reported among military troops stationed at high altitude (9). Increased infant mortality rates among high altitude natives due to respiratory infections have also been reported (2). Soviet investigators report an increased incidence of lung infections among natives residing above 2,600 meters (5). The lack of diseases transmitted by mosquitoes at higher elevations and increased sensitivity of some pathogenic organisms to reduced oxygen tensions are just two factors, however, which

confound the interpretation of epidemiologic studies from mountain environments (10). Furthermore, since individuals visiting high altitude environments are frequently exposed to multiple stressors including; cold, dehydration, novel infectious agents and poor hygiene, it has been difficult to ascribe an increase in infectious diseases exclusively to hypoxia-induced immune depression.

A large collaborative study entitled "Operation Everest II" provided an opportunity to investigate the influence of progressive hypoxemia on the human immune system. Seven healthy subjects were studied under controlled conditions during a 6 week hypobaric chamber exposure simulating an ascent from sea level to 29,000 ft (7,620 m). Multiple in vivo and in vitro parameters of immune function were investigated to determine; which aspect of the immune system is most sensitive to hypoxia-mediated influences, and identify possible mechanisms which may be responsible for altered immune responsiveness following exposure to this severe physiologic stressor.

MATERIALS AND METHODS

Study Protocol and Sample Collection

Subjects were 7 male volunteers age 21 to 31 years with a mean (±SE) height and weight of 183±3 cm and 89±4 kg

respectively. An eighth subject was withdrawn prior to the final blood sampling at 25,000 ft (7,620m) when he suffered an acute hypoxic syncopal episode. All subjects were in good health at the onset of the experiment as determined by medical history, physical exam and routine laboratory screening. Each subject gave written informed consent to participate in the study.

The subjects spent five days in the large hypobaric chamber at the U.S. Army Research Institute of Environmental Medicine in Natick, MA at sea level while baseline studies were performed. The chamber was then gradually decompressed over 28 days to an altitude of 25,000 ft (7,620m) while the subjects participated in numerous physiologic tests to evaluate adaptation to hypoxia (11). The chamber was decompressed further to 29,000 ft (8,839m) for periods of one to four hours to allow additional physiologic testing at this maximum altitude. Temperature and relative humidity were maintained at 20^{o}C and 35% respectively. The subjects were given ad libitum access to fluids, a nutritionally balanced diet and encouraged to exercise daily. The only administered medications included; the occasional use of acetaminophen for headaches, throat lozenges, and 2 subjects used triazolam as a hypnotic at extreme altitude.

In an attempt to assess potential effects of hypoxia on the immune system, we performed a variety of *in vitro* assays and some *in vivo* measurements of immunologic parameters. Forty mls of peripheral blood were collected from the fasting subjects

at 7:30 a.m. at sea level, 7,500 ft (2,286m) and 25,000 ft (7,620m) as indicated in Figure 1. From ficoll-hypaque isolated leucocytes we determined: the percentage of mononuclear cell phenotypes (B cells, monocytes and T cell subsets), Natural Killer cell cytotoxicity, mitogen-stimulated activation (protein synthesis), proliferation (thymidine uptake), interferon production, and immunoglobulin secretion. The concentration of circulating immunoglobulins, and antibodies to nuclear antigens were measured in plasma samples. Mucosal immune function was assessed by quantifying total protein, IgA and lysozyme levels in nasopharyngeal washings obtained weekly.

In Vitro Immunologic Assays

Mononuelear Cell Thymidine Uptake and Protein Synthesis

Peripheral blood was collected in 3 sterile heparin tubes (Becton-Dickinson, Rutherford, NJ), centrifuged at 400xg for 10 minutes and the plasma was stored at -20°C to -70°C. Mononuclear cells were isolated by ficoll-hypaque (Sigma, St. Louis, MO) density gradient sedimentation as previously described (12). The cells were cultured in RPMI 1640 (Gibco, Chagrin Falls, OH) containing 20% heat-inactivated fetal calf serum (HIFCS), (Biolabs, Northbrook, IL), 5% v/v 200 mM glutamine (Flow, Mclean,

VA), and 1% v/v antibiotic containing penicillin, streptomycin and amphotericin (Gibco). Final cell concentrations were determined with a ZH Coulter counter (Coulter Inc. Hialeah, IL) and adjusted to 1.0 x 10^6 cells/ml. Triplicate cultures containing phytohemagglutinin (PHA) 1 ug/ml, (Burroughs Wellcome, Greenville, NC) (PWM) 1/200 dilution (Gibco) or media alone were performed in 200 ul volumes using round bottom microtiter plates (Linbro, Flow Labs Inc.) The culture plates were transported in portable incubators (Millipore Corp., Bedford, MA) to the Center for Blood Research in nearby Boston for incubation at 37°C, 5% CO_2 and 100% humidity.

Protein synthesis was measured in 24 hour cultures following a three hour pulse with 2 uCi/well of ^{35}S-methionine (sp. act. 130 mCi/mM, Amersham, Arlington Heights, Ill.). Radioactivity in trichloroacetic acid precipitable protein was determined as previously described (13), after harvesting cells onto glass filter strips with a Mini-Mash II harvester (MA Bioproducts, Wallsville, MD).

Lymphocyte proliferation was measured in 72 hour cultures following a 2 hour pulse with 1 uCi/well of 3 H-thymidine (sp. act. 2mCi/mM, NEN, Research Products, Boston, MA) as previously described (12). The protein synthesis and thymidine uptake data are represented as the means of triplicate cpm/10^6 cells cultured.

Interferon Production

Triplicate cultures of 1.0 x 10^6 mononuclear cells in 1 ml of media described above were cultured with and without PHA in 5 ml plastic tubes (Falcon, Cockeysville, MD). After 72 hours in culture, the cells were centrifuged at 400xg for 15 minutes and 800 ul of the supernatant was transferred to plastic vials and stored at -70°C. All samples were thawed and assayed simultaneously at UTMB for interferon activity using a virus microplaque reduction assay as previously described (14,15). Briefly, monolayers of human aminon (WISH) cells were treated overnight in 96 well microtiter plates with dilutions of the culture supernatants and then challenged with vesicular stomatitis virus. The dilution at which there is a 50% reduction in the number of virus plaques is one unit of antiviral activity and the reciprocal of the dilution is the titer. The assay was standardized so that one unit equals one NIH reference unit.

Flow Cytometry Analysis of Mononuclear Cell Phenotype Markers

Ten mls of peripheral blood were collected in tubes containing EDTA (Becton-Dickinson) and ficoll-hypaque isolated mononuclear cells were stained by direct and indirect immuno-fluorescence with monoclonal antibodies. Briefly, 100 ul aliquots of 1 x 10^6 mononuclear cells were placed in plastic tubes

containing 25 ul of human AB serum (KC Biologicals, Lenexa, KY) and phycoerythrin-conjugated monoclonal antibodies (PE-MoAb) from Becton-Dickinson: anti-leu3a (helper/inducer), anti-leu2a (suppressor/cytotoxic), anti-M3 (monocyte), anti-Dr (B cell and monocyte) a PE control or anti-HLe1 (pan leukocyte). The pan leukocyte marker required a second step anti-mouse antibody conjugated to fluorescein isothionate (Becton-Dickinson). The cell suspensions were incubated for 30 minutes and washed twice following centrifugation at 300xg in PBS containing 1% v/v HIFCS and 0.05 M sodium azaide (Sigma). All steps were performed at 4^{o}C, and after the final wash, the cells were fixed in 1.5mls of 1% cold paraformaldehyde in normal saline. All samples were analyzed within one week at Johnson Space Center using an EPICS V flow cytometer (Coulter). The 5 watt argon ion laser (Coherent, Palo Alto, CA) was maintained at 488 nm and regulated to 500mW constant light output. Green fluorescence signals from samples and controls stained with FITC were analyzed on the logarithmic integrated "green" fluorescence channel using a 515 nm quartz interference filter and 590 nm long pass filter. A 560 nm dichroic filter and 590 nm long pass filter were used to collect logarithmic integrated "red" fluorescence signals from samples stained with PE. Ten thousand mononuclear cells were counted after gating on forward angle light scatter to exclude cell fragments, debris, and electronic noise. The percentage of cells stained with a specific MoAb was determined from the resulting histograms as previously described (16).

Natural Killer Cytotoxicity Assay

Mononuclear cells resuspended in RPMI 1640 containing 1% HIFCS, 1% antibiotic, 5mM Hepes (Gibco) and 2mM glutamine were placed into microtiter wells (Linbro plates). Triplicate samples of 200 ul volumes containing 2.5×10^5, 1.25×10^5, and 6.25×10^4 cells per well were cultured in media alone, 2 units of recombinant interleukin-2 (rIL 2, Amgen Biologicals, Thousand Oaks, CA) or 200 units of recombinant interferon alpha-2b (rIFN 2b, Intron, Schering Corp. Bloomfield, N.J.) to yield final effector-to-target cell (E:T) ratios of 50:1, 25:1, and 12.5:1 respectively. Mycoplasma-free K562 cells, an NK susceptible target cell line was derived from a patient with chronic myeloid leukemia. These cells were gently washed in PBS and 2.0×10^6 cells were labelled for 60 minutes with 100 uCi of ^{51}Cr (sp. act. 250-500 mCi/mgCr, Amersham) by incubation with constant shaking at 37°C. After washing with RPMI 1640 containing 10% HIFCS, 0.5×10^4 labelled K562 cells in 100 ul were added to each well containing effector cells which had been cultured for 24 hours. Following an additional 4 hour incubation, the plates were centrifuged at 120xg and 100 ul of supernatants were counted in an automatic Gamma Counter 1185 (LKB Instrumental Inc., Gaithersburg, MD) to determine the proportion of ^{51}Cr released. Control wells with labelled K562 cells cultured without effector cells or with detergent were used to determine the spontaneous and maximum release respectively. The percent cytotoxicity was calculated by the following formula (17): CPM Exp-CPM Spont./CPM Total-CPM

Spont. X 100 = % cytotoxicity.

The % cytotoxicity was converted to lytic units per 10^7 effector cells at 10% cytotoxicity ($LU/10^7$) using linear regression analysis thus reflecting the number of effector cells required to lyse the same percentage of target cells (18). With this method of data reduction, 1 lytic unit represents the reciprocal of the number of effectors required to achieve 10% lysis per 10^7 mononuclear cells.

In Vitro Immunoglobulin Production

Spontaneous and PWM stimulated IgG, IgA and IgM secretion was quantitated in triplicate 1 ml cultures. After 72 hours of culture in polystyrene tubes, 500 ul of fresh media was added to each tube containing 1.0×10^6 cells in 500 ul of RPMI 1640 complete media with 20% HIFCS (19). Following 9 days in culture, the cells were centrifuged at 300xg for 15 minutes and 800 ul of the supernatant was removed and stored at -70°C. All supernatants were thawed simultaneously and the quantity of immunoglobulins were determined by an ELISA assay. Round bottom 96 well microtiter polystyrene plates were coated with optimum concentrations of an Ig fraction of rabbit anti-human IgA, IgG and IgM serum (150 ul/well) (Accurate Chemicals and Scientific Corp., Westbury, NY) diluted to 3 ug/ml in 0.125 M borate buffered saline

solution. Following a 4 hour incubation at 37°C, the plates were stored at 4°C. The coated plates were then washed with 0.05% Tween 20-saline and 150 ul of samples diluted to 1/5, 1/25, and 1/125 with PBS-tween were added to triplicate wells. A standardized serum containing known concentrations of human IgA, IgG, and IgM (Calbiochem Behring Corp., LaJolla, CA) was used to develop standard curves for each immunoglobulin. The plates were then incubated for 2 hours at 20°C with continuous agitation followed by a washing with Tween 20-saline. Next, 150 ul of horseradish peroxidase conjugates of rabbit anti-human IgA (1/50), IgG (1/200), and IgM (1/200) (Accurate Chemicals and Scientific Corp.), were added to appropriate wells. This was followed by an additional 2 hour incubation at 20°C with continuous agitation and a final wash with tween-20 saline. The amount of enzyme bound to each well was determined by adding 150 ul of citrate buffer containing 2.5 ul/ml of 3% H_2O_2 and 0.1 mg/ml O-phenylenediamine to each well. The enzymatic reaction at 20°C was terminated after 1 to 2 minutes by adding 40 ul of 5N H_2SO_4. The optical density of the product was determined at 490 nm with an automated micro ELISA reader MR 580 (Dynetch Instruments, Alexandria, VA). The assays were arranged so that each separate plate contained supernatants from only one subject at all three altitudes and a standard curve for IgA, IgG, or IgM concentration. The concentration of immunoglobulin in each supernatant was then determined by plotting the enzyme

activity (OD 490) against the log of the immunoglobulin content of the standard or the dilution of the sample. The results were expressed as the amount of each immunoglobulin secreted per 1 x 10^6 mononuclear cells cultured.

In Vivo Immunologic Parameters

Nasal Pharyngeal Secretion Assay

Weekly nasopharyngeal washings (figure 1) were obtained by instilling 5 ml of sterile PBS into each nasal chamber of the seated subjects while hyperextending their neck. The subjects then flexed their necks and the washings were collected in plastic cups. The specimens were transferred to polypropylene vials (Nunc, Roskille, Denmark) and stored at -70°C. Thawed nasal wash supernatants were concentrated 5-fold by a Minicon B15 concentrator (Amicon Corp, Danvers, MA) which retains molecules > 15,000 MW. Each specimen was tested for the presence of blood by Hemastix (Ames Co., Elkhart, IN). Total protein was measured by a peptide binding assay (Bio-Rad Protein Assay, Bio-Rad, Richmond, CA) based on the differential color change of Coomassie G-250 and compared to a standard curve of bovine plasma albumin and gamma globulin (20). Total IgA content was measured by quantitative single radial immunodiffusion on low level IgA immunoplates (Kallestad Labs, Austin, TX) (21). Lysozyme content was measured

by a modified bacteriolytic assay (22). Briefly, lysoplates were prepared using 1.0% sodium chloride, 0.1% sodium azide, and 0.05% Micrococcus lysodeikticus (Sigma). This medium was pipetted into standard (15mm x 100mm) plastic petri plates (20 ml/plate) and stored in sealed plastic bags at 4°C. A series of lysozyme standards (1,000 to 10,000 ug/ml were made by diluting muramidase (from human milk, Sigma) with 0.2M phosphate buffer (pH 6.3) containing 1.0% sodium chloride. Four standard filter paper discs (6.5 mm diameter) were placed on each lysoplate. Three microliters of each standard or nasal wash supernatant fluid were deposited onto separate filter paper discs. Duplicate determinations were performed on different plates. The plates were incubated for 24± 0.5 hours at 35°C in a humidified chamber. Following this incubation, the diameter of the zone lysis was measured to the nearest 0.5 mm. A standard curve was developed from linear regression analysis of the zone size vs the logarithm of the lysozyme concentration. The lysozyme concentration of each nasal wash supernatant was determined from this standard curve.

Plasma Immunoglobulins and ANA Titers

Plasma samples collected in EDTA were stored at -70°C, thawed simultaneously and analyzed by rate nephelometry for IgA, IgG, and IgM concentrations using the ICS Analyzer II (Beckman).

These plasma samples were also diluted 1:20 and 1:40 and analyzed by indirect immunofluorescence microscopy to identify the highest dilution which allowed a positive nuclear fluorescence profile to be identified. The HEp-2 cell line was the substrate used for detecting antibodies against nuclear antigen. Based on prior samples from normal donors, a titer of 1:80 or higher was considered significantly elevated.

Data Analysis

All data were entered into the UTMB CRC Clinfo computer (Bolt, Beranek and Newman Inc., Cambridge, MA). Normally distributed data were analyzed by analysis of variance with repeated measures (BMDPR Statistical Software Inc., Los Angeles, CA.). Differences between altitude exposures were identified using multiple comparison methods with proper standard error calculated from analysis of variance. The Wilcoxon Signed Ranks Test was used for data not normally distibuted and p values less than 0.05 were considered significant.

RESULTS

In Vitro Immunologic Assays

Flow cytometry analysis of mononuclear cell subsets prior to culture are displayed in Table 1. The percentage of M3 positive

cells (monocytes) was significantly elevated at 25,000 ft (7,620m) with a range of 13-32% whereas the range of M3 positive cells was 5-13% at sea level and 7,500 ft (2,286m) and 10-12% for the alternate subject on three occasions. In contrast, the percentage of T cell subsets and B cells were uninfluenced by altitude.

Following 4 weeks of progressive hypoxemia, a significant reduction in PHA-stimulated in vitro mononuclear cell proliferation at 72 hours was detected by tritiated thymidine uptake (figure 2). Each individual subject demonstrated a reduced PWM and PHA stimulated response at 25,000 ft (7,620m) compared with 7,500 ft (2,286m) values but PWM responses were similar at sea level and 25,000 ft. The mean unstimulated ^{3}H-thymidine values were $6 \pm 1 \times 10^{3}$ cpm for baseline altitudes whereas $8 \pm 1 \times 10^{3}$ cpm at 25,000 ft. was only significantly elevated when compared with data from 7,500 ft. (2,286m). The results from our alternate subject who had blood drawn at identical times did not indicate significant changes in the assay as mitogen responses were similar at each altitude exposure (PWM; 62,785 vs 68,248 vs 64,656, and for PHA; 171,125 vs 147,338 vs 157,493 cpm/10^{6} cells cultured). Significant differences were also detected within the first 24 hours of culture with PWM and PHA stimulated protein synthesis (figure 3). Unstimulated cells from subjects at 7,500 ft and 25,000 ft exhibited a reduced ability to synthesize new polypeptides only when compared with sea level values. Results from our subject not exposed to altitude

indicated the reduced responses observed at 25,000 ft. were not due to changes in the assay (unstimulated; 3,900 vs 4,790 vs 4,030, PWM; 4,234 vs 7,250 vs 6,869, PHA; 13,750 vs 15,275 vs 13,281 cpm/10^6 cells cultured). Therefore, significant reductions in mitogen stimulated mononuclear cell activation and proliferation responses were detected from subjects following exposure to an altitude of 25,000 ft (7,620m).

PHA stimulated interferon production from each subject at altitude is presented in Table II. The greater intersubject variations observed at 25,000 ft. prevented statistical differences from being observed between altitude exposures. The results obtained at high altitude, however, were quite remarkable since PHA-stimulated lymphocytes from 3 subjects (number 5,6, and 7) produced greatly reduced amounts of IFN. Unstimulated lymphocytes from all subjects produced very small amounts of IFN which ranged between <10 and 30 units/ml and were uninfluenced by altitude exposure (data not shown in table II). Interferon production from the individual not exposed to altitude was < 10 units for unstimulated and 300 units/ml for PHA stimulated lymphocytes on each of 3 sampling periods and were analyzed simultaneously with the other subjects. Therefore in contrast to a uniformly reduced PHA stimulated thymidine uptake response following exposure to 25,000 ft (Figure 2), lymphocytes from 2 subjects had greatly increased IFN production whereas 3 subjects had reduced IFN production compared with values obtained at sea level and low altitude.

Natural Killer cell cytotoxicity results from sea level and high altitude during spontaneous, rIL-2 and rIFN 2 augmentation were: 103±54 vs 53±19, 408±116 vs 1,480±637 and 799±555 vs 494±184 lytic units/10^7 cells, (sea level vs 25,000 ft) respectively. Data were not available at 7,500 feet. There were however remarkable individual differences observed among three subjects at 25,000 ft (7,620m) since one subject had no augmentation in response to rIFN or rIL 2 whereas two other subjects' rIL-2 augmented killing greatly exceeded their sea level values. No statistical differences, however, were identified when cytotoxicity data were converted to lytic units for the entire group (Wilcoxon Signed Ranks Test).

Data in figure 4 represents in vitro polyclonal B cell function at the various altitudes. No significant differences were observed after 9 days of culture in spontaneous or PWM stimulated IgA, IgG or IgM production at any altitude despite reduced PWM stimulated protein synthesis and thymidine uptake at 24 and 72 hours following exposure to 25,000 ft (7,620m) vs 7,500 ft (2,286m) (figures 2 and 3).

In Vivo Immunologic Parameters

The results of weekly nasal wash IgA and lysozyme levels during 6 weeks of exposure to progressive hypoxemia are displayed in figure 5. The data are expressed as a ratio of total protein to IgA and lysozyme in ug/ml since the recovery volume of the instilled saline wash solution varied between different subjects

and between sequential collections from the same subjects. No significant effect of altitude exposure on either IgA or lysozyme levels in nasal wash was detected by ANOVA.

The measurement of plasma immunoglobulins reported in table III indicate significant elevations in IgM, and IgA but not IgG levels at 25,000 ft (7,620m). There was, however, no evidence of increased in vivo production of autoantibodies against nuclear antigens since no subject's plasma exhibited a significantly positive immunofluorescence staining pattern despite testing at very low dilutions (1:20 and 1:40).

DISCUSSION

The results of this study indicate that 4 weeks of progressive hypobaric hypoxia simulating an altitude exposure of 25,000 ft (7,620m) induces alterations in several in vitro and in vivo immunologic parameters. Operation Everest II provided an ideal opportunity to evaluate which aspect of the human immune system is most sensitive to in vivo influences following exposure to a severe physiologic stressor. Our cell culture conditions were optimum for PWM-stimulated in vitro immunoglobulin production yet also supported other mitogen stimulated mononuclear cell responses including protein synthesis, proliferation, and interferon production (19). The mitogenic lectins, PHA and PWM, were used to activate resting monocytes and lymphocytes since very few of these cells can be activated in vitro by a single antigen and mitogen stimulated biochemical

activation events leading to in vitro differentiation and immune effector cell function are quite analogous to antigen stimulated responses (23). Similar in vitro activation assays have successfully been employed to study normal immune regulation and elucidate multiple cellular and biochemical defects associated with a variety of diseases in humans (24,25).

Our findings of uniformly blunted PHA stimulated thymidine uptake and protein synthesis at extreme altitude, suggest that T cell function in healthy subjects may be impaired following continous exposure to progressive hypoxemia despite a gradual ascent to facilitate acclimitization. Three separate Soviet studies also reported reduced in vitro human T cell mitogenic responsiveness (3,200-3,800m) which returned to "normal" after 25 to 30 days at altitude (5-7). Impaired T cell mitogenesis has also been reported following exposure to other environmental or psychological stressors including; space flight (12,16,26), bereavement (27), academic examinations (28) and several other stressors (29). In none of these prior studies, however, was a reduction of the magnitude reported in our study observed.

We obtained two values at low altitude to serve as baselines (assay variability) and to identify any influence of "stress" per se associated with participating in this protocol. All subjects underwent several invasive procedures (right heart catheterization and muscle biopsies) between sea level and 7,500 ft (2,286m). Using this identical thymidine uptake assay, we were

previously unable to document changes in PHA or PWM stimulated lymphocyte proliferation among 28 volunteers who participated in an acute decompression chamber study despite 1/4 of the subjects reporting some symptoms of decompression sickness (30). Therefore, a 33% reduction in PHA-stimulated thymidine uptake in addition to a 50% reduction in protein synthesis at 25,000 ft with these assays indicates that circulating human mononuclear cells are substantially depressed in their ability to become activated in vitro following exposure to this severe physiologic stressor.

In contrast to T cell responses, B cell functions were not depressed in this study. Despite blunted PWM-stimulated in vitro protein synthesis at extreme altitude, IgG, IgA or IgM secretion into culture supernatants after 9 days was not depressed. The elevated plasma IgG and IgM levels at altitude may reflect in vivo polyclonal expansion. However, unstimulated and PWM stimulated in vitro Ig production were not increased. Increased anti-cardiac antibodies were reported in one Soviet study following exposure to altitude (1) but we were unable to detect even low titers of antinuclear antibodies despite each of our subjects receiving multiple muscle biopsies. In vivo B cell function was unimpaired in two Soviet studies reporting normal responses to vaccinations (2,5).

Mucosal immunity as measured by nasal wash IgA and lysozyme levels was not impaired despite exposure to 29,000 ft. These findings support the interpretation of data obtained from Antartica subjects, that reduced IgA and lysozyme

levels resulted from the combined stressors of cold, isolation, and reduced humidity (31). Hypoxia per se, due to the reduced barometric pressure at the South Pole (simulating an altitude of 10,000 ft) probably was not a contributing factor to blunted mucosal immunity.

The NK cytotoxicity assay and PHA stimulated interferon production revealed greator inter-subject variance at extreme altitude than the other simultaneous in vitro immune assays. This underscores the heterogeneity of in vitro cellular responses following exposure to a severe physiologic stressor.

Complex immunoregulatory mechanisms may be responsible for altered in vitro lymphocyte responses from subjects after exposure to stressful environments. Acute hypoxia causes alterations in a number of circulating hormones which could influence mononuclear cell function (32-34). In addition to the immunosuppressive effects of glucocorticoids (35), recent studies indicate that the nervous system modulates immune effector cell function via common neuropeptide hormones and cell surface receptors shared by the immune and neuroendocrine systems (36-37).

The observed increase in monocytes following exposure to 25,000 ft may be analogous to the increase reported following exposure to other stressors including psychological and physical exercise (38). This increase in monocytes cultured could have been responsible for our observed in vitro alterations at

high altitude since the contribution of monocytes in regulating in vitro immune responses is well documented (13,19,39). However, since reduced thymidine uptake has also been observed following space flight (16) and academic examinations (26) without an increase in cultured monocytes, the blunted proliferation responses at 25,000 ft were probably not directly related to culturing increased numbers of monocytes. Furthermore, since monocytes contribute greatest to mitogen stimulated protein synthesis at 24 hours (compared with B cell and T Cell responses), an increase rather than a decrease in the protein synthetic response should have been observed unless the cultured cells were less responsive to early mitogenic activation signals following hypoxic stress (13).

Since the human immune system has multiple self-regulating networks it is hazardous to extrapolate results from in vitro generated data to complex in vivo systems. However, rodents exposed to hypoxia (4,000m-7,200m) have increased susceptibility to fatal bacterial infections following challenge with multiple pathogens (40-44). Two recent studies have also demonstrated that blunted in vitro T cell responsiveness following traumatic injuries were associated with increased complications due to infections (45,46). Therefore, our finding of lowered in vitro cellular responses to mitogenic activation may also indicate reduced in vivo immune competence in humans exposed to hypoxia.

This study indicates that human *in vitro* immune responses are modulated by exposure to hypobaric hypoxia simulating an ascent to 25,000 ft under controlled conditions. Exposure to this environmental stressor appears to alter normal *in vitro* immune regulation. Future studies could utilize hypoxia as a physiologic stressor to define mechanisms of neuroendocrine-mediated modulation of human immune responses following exposure to stressors.

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Figure 1: Rate of ascent and corresponding simulated altitude exposures in the decompression chamber during Operation Everest II. Three blood samples (circles) and seven nasal wash specimens (triangles) were collected at the various altitudes indicated.

Figure 2: Tritiated thymidine uptake by in vitro mononuclear cells after 72 hours in culture are displayed. Results from unstimulated (open bars), PHA (hatched bars) or PWM (solid bars) stimulated cells were obtained following sea level, 7,500 ft., and 25,000 ft altitude exposures. Values are group means $\pm$ SE of cpm per 10^6 mononuclear cells cultured and * indicates significant differences between altitudes at $p < .05$ by ANOVA.

Figure 3: The results of ^{35}S methionine incorporation into TCA precipitable protein after 24 hours in culture are displayed. Results from unstimulated (open bars), and PHA (hatched bars) or PWM (solid bars) stimulated mononuclear cells were obtained following sea level, 7,500 ft. and 25,000 ft. altitude exposures. Values represent group means $\pm$ SE of cpm per 10^6 cells cultured at sea level, 7,500 ft. and 25,000 ft.

altitude exposures. * indicates a reduction in mitogen free protein synthesis at 7,500 ft vs sea level values and PWM and PHA stimulated protein synthesis at 25,000 ft. compared to sea level and 7,500 ft altitude values, $p < .05$ by ANOVA.

Figure 4: The results of spontaneous and PWM stimulated in vitro production of IgA, IgG and IgM into supernatants following 9 days of culture are displayed. Data represent group means ± SE of each immunoglobulin in ng per culture determined by ELISA in supernantants from unstimulated (open bars) or PWM stimulated (solid bars-IgA, hatched bars-IgG and striped bars-IgM). No statistical differences were observed between samples cultured following exposure to sea level, 7,500 ft., or 25,000 ft. altitudes.

Figure 5: The results of lysozyme and IgA levels in nasal wash collections obtained at each altitude exposure are indicated. Data represent group means ± SE of ratios of IgA (triangles) and lysozyme (circles) to total protein in wash specimens.
No statistical differences were observed between any altitude exposure and lysozyme or IgA levels by ANOVA.

Table I

EFFECT OF SIMULATED ALTITUDE EXPOSURE ON PERIPHERAL BLOOD MONONUCLEAR CELL PHENOTYPES

	Sea Level	7,500 Ft.	25,000 Ft.	ANOVA
M_3+ cells (monocytes)	9 ± 1	8 ± 1	19 ± 3	<.05
Dr+ cells (monocytes & B cells)	14 ± 3	15 ± 3	27 ± 2	<.05
B cells (Dr+ cells minus M_3+ cells)	6 ± 3	9 ± 3	9 ± 3	NS
Leu3a+ cells (T helper/ inducer)	42 ± 4	44 ± 2	36 ± 6	NS
Leu2a+ cells (T suppressor/ cytotoxic)	24 ± 3	23 ± 2	22 ± 4	NS

Data are mean + S.E.M. of the percentage of circulating peripheral blood mononuclear cells positive for cell surface antigens.

Table II

EFFECT OF SIMULATED ALTITUDE EXPOSURE ON
IN VITRO PHA-STIMULATED INTERFERON PRODUCTION

Subjects	Sea Level	7,500 Ft.	25,000 Ft.
1	3,000	3,000	10,000
2	300	300	3,000
3	1,000	600	300
4	300	100	300
5	3,000	1,000	300
6	1,000	300	30
7	300	200	30
Mean ± S.E.M.	1,271 ± 425	785 ± 357	1,994 ± 1,287

Data are triplicate means of IFN units/ml secreted into cell culture supernatants after 72 hours.

Table III

EFFECT OF SIMULATED ALTITUDE EXPOSURE ON
PLASMA IMMUNOGLOBULIN LEVELS

	Sea Level	7,500 Ft.	25,000 Ft,	ANOVA
IgG (63-1,349 mg/dl)	1,033 ± 58	996 ± 43	1,011 ± 46	NS
IgM (56-352 mg/dl)	122 ± 17	120 ± 17	163 ± 22*	<0.005
IgA (70-312 mg/dl)	154 ± 21	147 ± 22	181 ± 25*	<0.01

Data are group means ± S.E.M. for 7 subjects at each altitude. Normal values appear in parenthesis. Statistically different values from sea level and 7,500 feet are indicated by *.

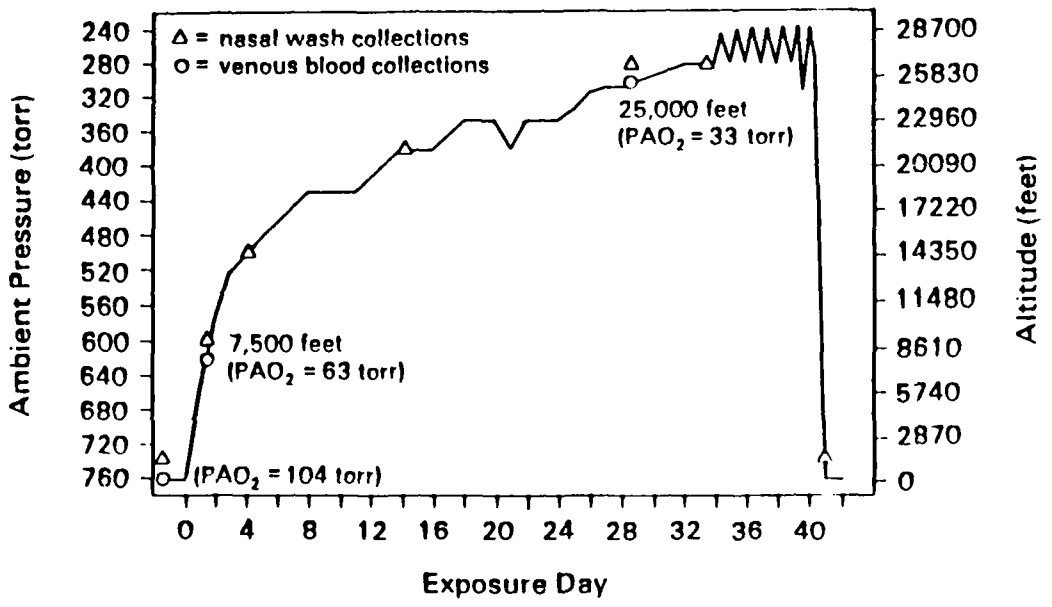
Δ = nasal wash collections
o = venous blood collections
25,000 feet
(PAO2 = 33 torr)
7,500 feet
(PAO2 = 63 torr)
(PAO2 = 104 torr)
Ambient Pressure (torr)
240
280
320
360
400
440
480
520
560
600
640
680
720
760
Altitude (feet)
28700
25830
22960
20090
17220
14350
11480
8610
5740
2870
0
0
4
8
12
16
20
24
28
32
36
40
Exposure Day

Figure 1

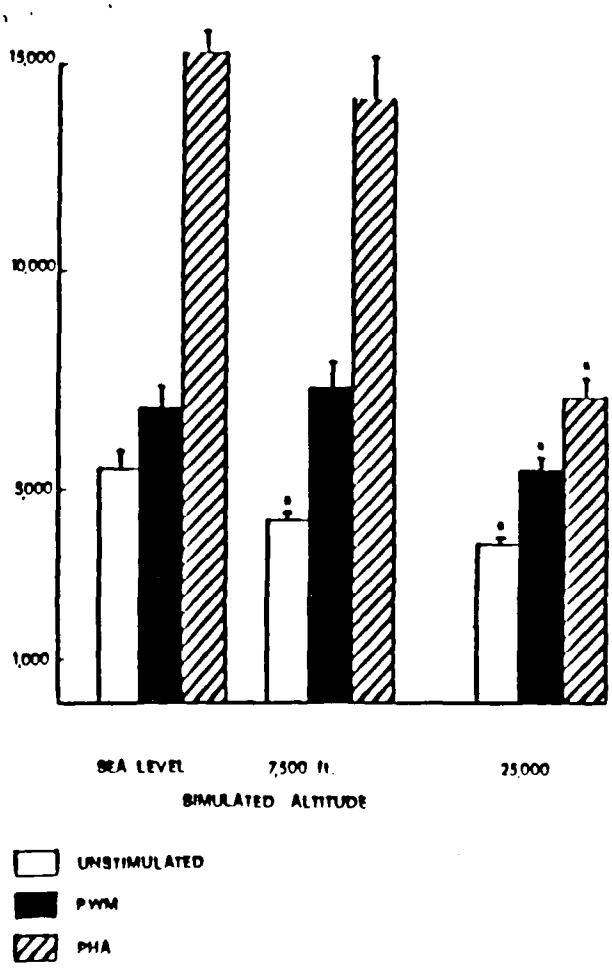

Figure 3

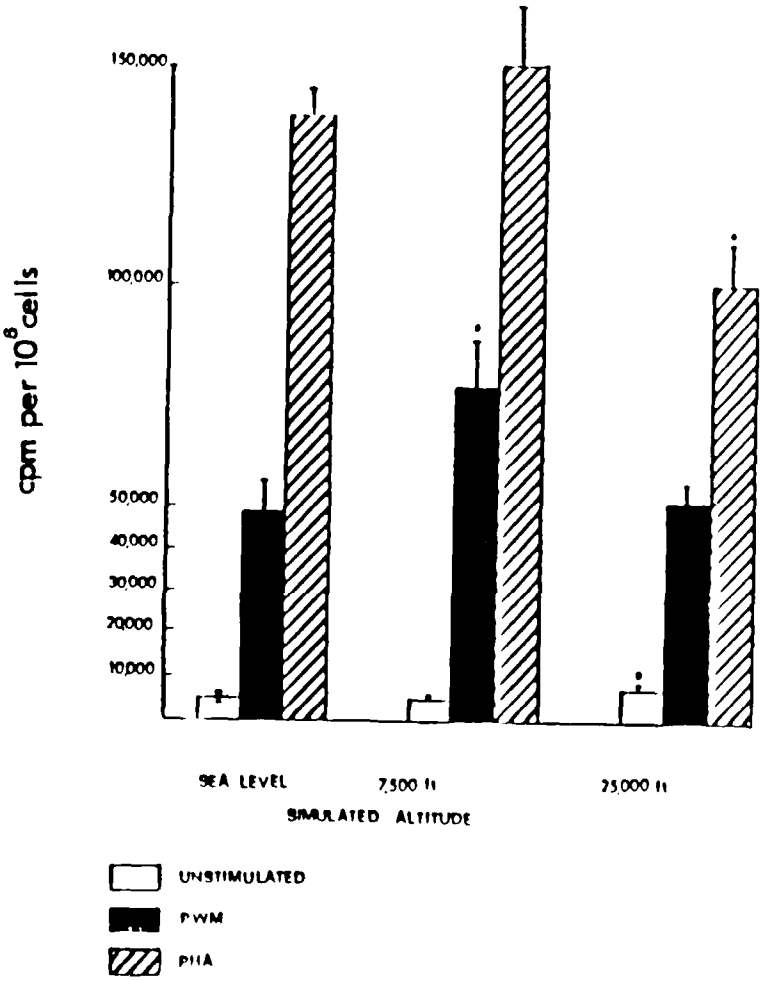

Figure 2

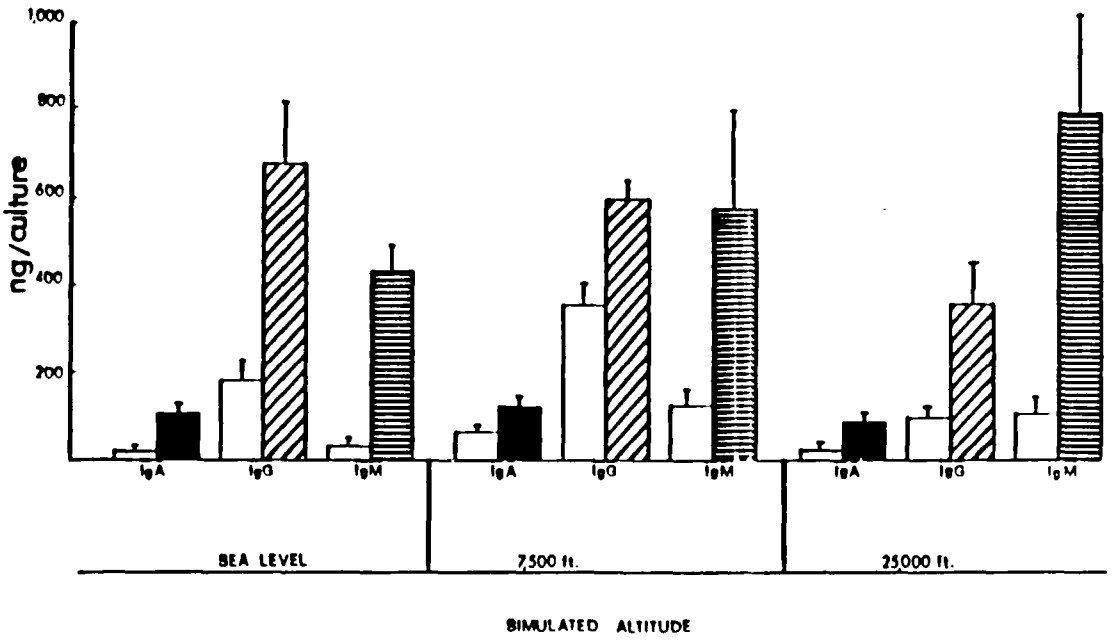

Figure 4

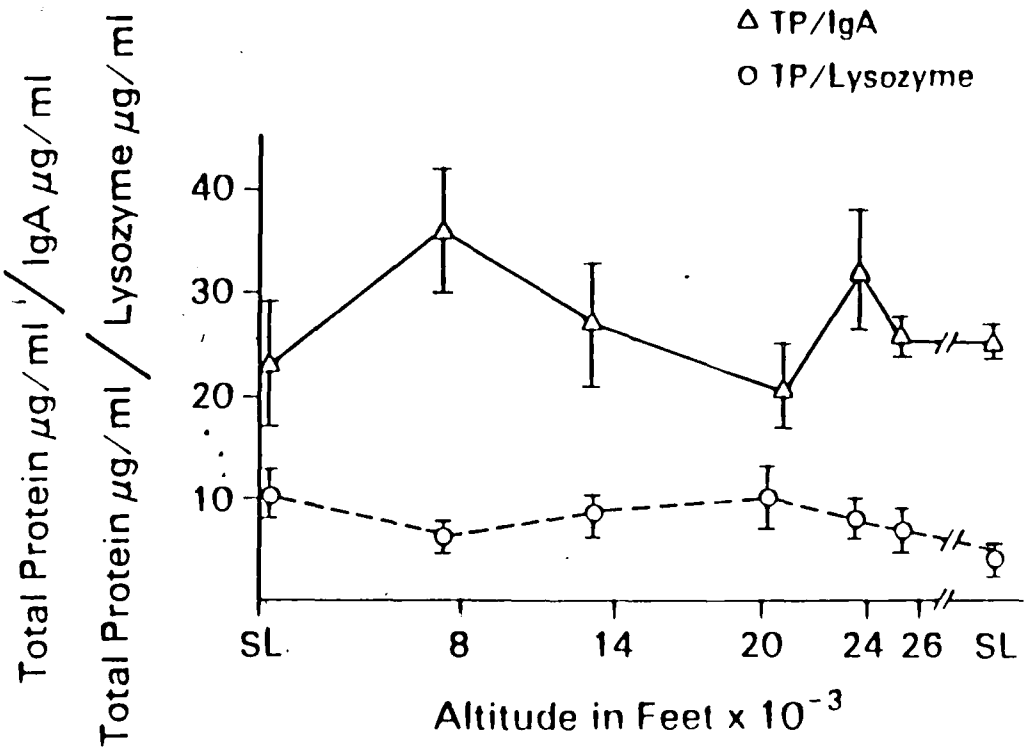

Figure 5

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